Gardening behaviour of *Sicydium punctatum* (Gobioidei: Sicydiinae): *in vitro* experiments in the context of chlordecone pollution in Guadeloupe Island rivers

by

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Abstract. – To fight the banana weevil, organochlorine insecticide was used in Guadeloupe, leading to a contamination of the rivers by chlordecone, for which the molecules are long lasting and persist in the environment although its use was banned in the 90s. In the rivers, Sicydiinae gobies of the genus *Sicydium* represent key species of the ecosystem. They are herbivorous and feed on the epilithic biofilm by scraping it off the rocks. We undertook *in vitro* experiments in order to obtain preliminary results on the effect of the pollutant on the behaviour of *Sicydium punctatum* Perugia, 1896, and on its food source. It seems that the pollutant has no effect on the social behaviour of this species. However, we noted that in polluted conditions, the biofilm composition is altered, depriving *S. punctatum* of its favoured diatom species, which *S. punctatum* usually "grows" by having a gardening behaviour.

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Résumé. – Étude *in vitro* du comportement de *Sicydium punctatum* (Gobioidei: Sicydiinae) dans le cadre de la pollution des rivières de Guadeloupe au chlordécone.

Les pesticides organochlorés, utilisés contre le charançon du bananier, ont contaminé les rivières de Guadeloupe au chlordécone, dont les molécules persistent encore malgré l'interdiction de son utilisation depuis les années 90. Dans les rivières, les Sicydiinae, des gobies du genre *Sicydium*, représentent des espèces clés de ces écosystèmes. Ils sont herbivores et broutent le biofilm qui se développe sur les roches au fond des cours d'eau. Nous avons réalisé des expériences *in vitro* afin d'obtenir des premiers résultats sur l'effet du polluant sur le comportement de *Sicydium punctatum* Perugia, 1896, et sur sa source de nourriture. Il semble que le polluant n'agisse pas sur le comportement territorial de cette espèce. En revanche, nous constatons que dans des conditions polluées, la composition du biofilm est altérée, privant *S. punctatum* de ses espèces favorites de diatomées, qu'il "cultive" grâce à un comportement jardinier dans des conditions non polluées.

Key words
Sicydiinae
Sicydium punctatum
Guadeloupe
Chlordecone
Diatom
Gardening behaviour

In the French Antilles, the Islands of Guadeloupe and Martinique are currently facing a large-scale ecological and sanitary crisis. A massive contamination by organochlorine (OC) molecules, formerly used in pesticides for banana plantations, was discovered at the beginning of the 2000's; it infiltrated and contaminated all the ecosystems (Coat *et al.*, 2011). The most worrying organochlorine (OC) molecule is the chlordecone (CLD) (Kepone®, Curlone ®), which undergoes little to no degradation in the natural environment. The entire aquatic ecosystems, marine or freshwaters, are affected and the latter is particularly contaminated with world record values (Coat and Monti, 2006). This pollution is comparable to that of the river James and the Chesapeake Bay, which were polluted in the 1980s due to an incident in a nearby plant producing the pesticide Kepone®; consequently, fishing and consumption of fish and crustaceans has been prohibited for years in the biggest estuary of the United States. For the last ten years in Guadeloupe, the priorities were defined to identify the most contaminated species and their level of contamination, to be able to protect the populations and to prevent and reduce exposure. The first step is to understand the mechanisms of the liberation of this pollutant in the environment and the consequences.

The rivers of these insular ecosystems have numerous specificities linked to the prevalence of migratory species in the macrofaunal biodiversity, many species are diadromous, *i.e.* they migrate from freshwater to seawater at different moments of their life cycle. The freshwaters are inhabited by sympatric Sicydiinae gobies, *Sicydium punctatum* Perugia, 1896 and *S. plumieri* (Bloch, 1786). These common species are adapted to the conditions of strong hydrological and climatic variations (Monti *et al.,* 2010). They feed,

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grow and reproduce in the freshwaters; larvae hatch in the river and undergo a downstream migration towards the sea. After a while at sea, they recruit back to rivers and during their upstream migration, they settle in a given territory and reproduce (Keith, 2003; Blob *et al*., 2006; Maie *et al*., 2007); thus the adults of *Sicydium* species are good spatial integrators. Juveniles of this genus are harvested in the estuaries of Caribbean rivers when they recruit and constitute fry fisheries in this region (Fièvet and Le Guennec, 1998; Bell, 1999). The two species are herbivorous, they scrape epilithic biofilm and diatoms off the substrate to feed (Watson, 2000; Monti *et al*., 2010). Given the level of contamination of the epilithic biofilm (Monti, 2005), they are directly impacted by chlordecone pollution.

To obtain information on the level of contamination of the ecosystems without having to make numerous and expensive chemical analyses, the French National Agency for Research (ANR) selected a program on the search for innovative bioindicators of CLD pollution as well as on the evaluation of the modifications in epilithic biofilm structures and functions induced by high CLD contaminations in Caribbean freshwaters (CHLORINDIC program, 2012-2016). A part of this multi-team research program included the study of the effects of the contamination on a target species, *S. punctatum*. The preliminary results presented here included investigations of modifications in microalgal composition of feeding territories and in behaviour of *S. punctatum*.

Several hypotheses were put forward: (i) the pollutant induces changes in the gardening behaviour of *S. punctatum,* (ii) the "gardens" are mainly maintained by dominant males, and (iii) diatoms found in the epilithic biofilm are the main source of food influencing grazing.

MATERIAL AND METHOD

To analyse the gardening behaviour of *S. punctatum*, we undertook *in vitro* experiments, from the 7th of January to the 18th of February 2013.

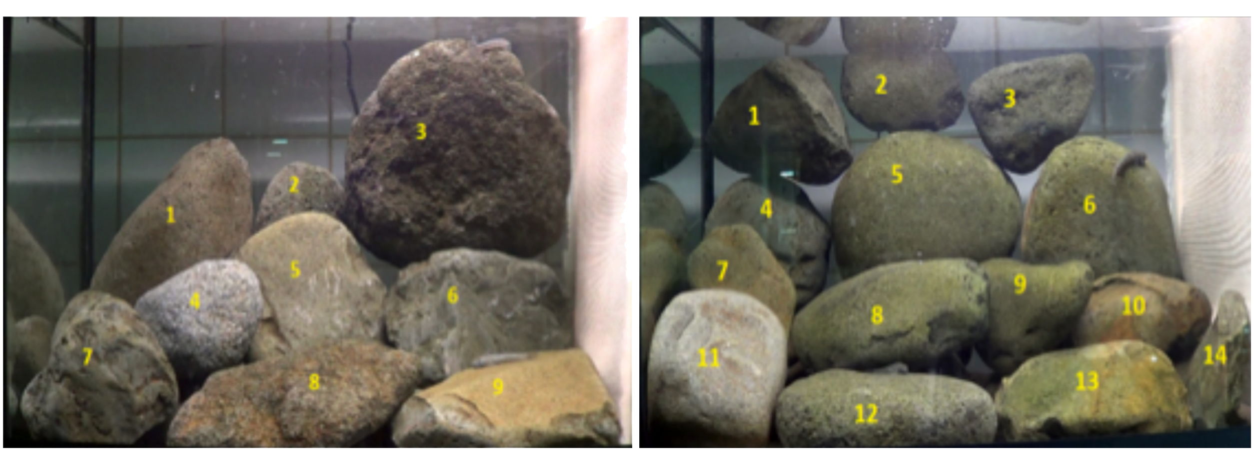
Experimental protocol

To install the *in vitro* experiment, water was sampled at two sites of the Pérou River, downstream, a polluted site ([Chlordecone] = 0.729 $mg.L^{-1}$), and upstream, above the banana plantations, thus free of chlordecone pollution. The CLD concentration was measured during the ANR Chlorindic program. Rocks covered in epilithic biofilm were also sampled and brought back to the laboratory in a wet environment and with the biofilm facing up in order not to damage the biofilm during transport. Three fish per site were sampled by electric fishing (Portable Dekka, *Gerätebau*, Marsberg, Germany), with at least one male in each group.

In the laboratory, two 110 L tanks were installed; one for the non-polluted environment, the other for the polluted environment. Each of them was filled with the water samples collected at each site and the rocks were installed in each tank in such a way as to maximise the size of the territory. Finally the three fish were put in their respective tank. Each rock in each tank was numbered to facilitate observation (Fig. 1). Moreover, a protected area was created in each tank to prevent fish from grazing it in order to compare grazed and protected biofilm at the end of the experiment.

Using an electric timer, a day/night cycle was established, as close as possible to natural conditions. A digital camera (Sony HDR CX-190E) was used to film fish activity during the day. Films were then analysed and several behavioural components were measured:

- total time per fish spent on each rock
- total time per fish spent grazing on each rock
- behavioural analysis



Non-polluted tank Polluted tank

Figure 1. – Non-polluted and polluted tanks for the *in vitro* experiment. The rocks were numbered in order to locate preferential cobbles chosen by the fish.

The epilithic biofilm overall structure was analysed using a BentoTorch (BBE Moldaenke, Germany) adapted for phytobenthos measurements and fast quantification of green algae, cyanobacteria and diatoms on stone surfaces. This instrument uses the fluorometric characteristics of the different algal pigments in the intact cell. The amount of red light fluorescence arising from excitation gives a quantitative estimate of the algal density and its classification. Results will be expressed as concentration of green algae, concentration of diatoms and concentration of cyanobacteria, in µg chl-a/cm^2, with a resolution of 0.2 µg chl-a/cm^2. Three measures per rock were taken at the end of the experiment allowing us to calculate a mean value for the biofilm composition in each tank.

All statistical analyses were undertaken using the MASS package in R 2.15.2 (Venables and Ripley, 2002).

Finally, at the end of the experiment, the three fish in each tank were sacrificed and the digestive track (DT) was collected from the mouth to the anus. Because one DT is not enough to analyse the diatoms in the gut, the three DT were pooled, giving one sample per tank. The epilithic biofilm in each tank was collected by scraping the surface of the submerged rocks with a brush. The samples collected from each cobble were pooled, giving one biofilm sample per tank. Each sample (biofilm sample and DT sample) was fixed in buffered formaldehyde at a final concentration of 10%. The preparation and mounting of diatoms were made in accordance with standard NF T 90-354 for both the pooled DT and for the biofilm samples. Identification of diatoms being based on microscopic examination of the siliceous frustules, samples were treated with hot concentrated hydrogen peroxide (H_2O_2 30%) to eliminate protoplasm and, when appropriate, with hydrochloric acid (removal of carbonates). After drying, the diatoms were mounted in refractive resin, Naphrax (Northern Biological Supplies Ltd, England – Refractive index = 1.74) and then identified. The counting protocol and identification of diatom valves is defined by the European Standard EN 14407 2004 and Stoermer *et al.* (1996).

RESULTS AND DISCUSSION

Epilithic biofilm is the most perennial component in river systems, especially young oligotrophic tropical rivers, making them important as bases of food webs; it is a major component at the basis of the diet of most of aquatic organisms in these regions (Burns and Keith, 2000; Coat *et al.,* 2009; Lefrançois *et al.,* 2010).

Gobies contribute most to the diversity of fish communities in the Caribbean insular systems (Keith, 2003; Lord *et al.,* 2010; Monti *et al.,* 2010). Adult freshwater *Sicydium* are mainly herbivorous but their diet changes between larval (carnivorous), juvenile and adult (herbivorous) stages; this diet shift is a key element for the survival of the species in the river (Schoenfuss *et al.*, 1997; Keith *et al.*, 2008). Metamorphosis of feeding structures from the offshore larval phase is vital for the survival of the fish once it enters freshwater. These changes are essential to begin feeding on biofilm and to migrate upstream which is the most important event in this type of life cycle, as it permits the colonisation of the watershed, the maintaining of the equilibrium between the fish communities and the energy transfers along species in the river. The timing and direction to the post-larval and juvenile upstream migration, towards the adult habitat, and mechanisms required for the success of this migration remain mostly unknown. However, some factors seem to control the upstream migration including (i) the trophic resources along the river (Keith and Lord, 2011), and (ii) the chemical composition of the biofilm and its exopolysaccharide matrix (EPS) through the release of dissolved free amino acids from biofilms in stream (Ishizawa *et al.,* 2010).

Six hours of film per tank were analysed. After sexing the individuals, there were one male and two females in the non-polluted tank and two males and one female in the polluted tank.

Evidence of a male dominant behaviour

Films show that in both tanks, one male shows a dominant behaviour and the other two fish were dominated, whether they were male of female. There were very few interactions between the dominant and the dominated. When there were any, fish were mainly aggressive, and the dominant was particularly aggressive towards the other two, chasing them away from its preferred cobbles. In both tanks, the three individuals were never seen on the same cobble at the same time.

Evidence of preferential cobbles

First of all, in both the polluted and the non-polluted tanks, there was evidence of preferential cobbles, *i.e.* the time spent in different areas of the tank varied. The time spent on each cobble was measured both for the dominant male and for the two dominated specimens. The time spent on each cobble was divided into the time spent grazing and the time spent being inactive. Results are illustrated in figures 2 and 3. They show that even though dominated individuals have different preferred cobbles from the dominant, all three individuals graze on the same cobbles. This result implies that the gardening behaviour is taken care of by the dominant specimen, as its preferred cobbles are the ones it feeds on. The dominant male probably maintains the growth of preferred microalgae, which the other specimens also benefit from. The only time the dominated specimens could graze on the "cultivated" cobbles was when the dominant was hiding beneath the cobbles.

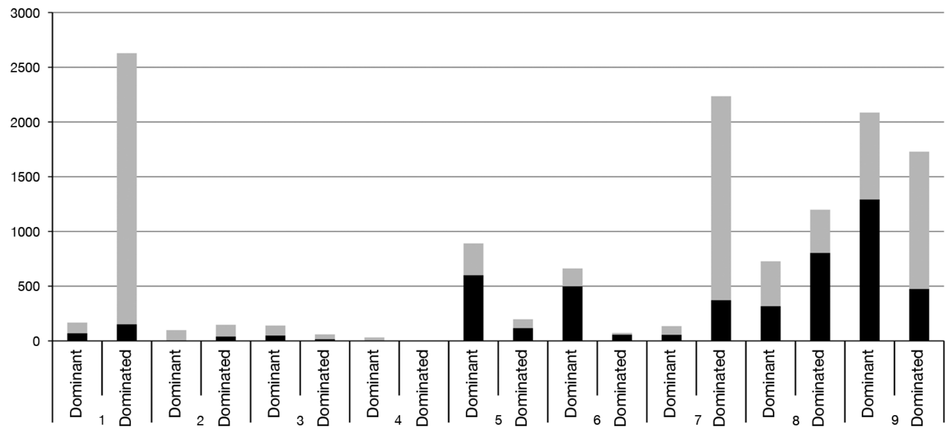


Figure 2. – Time in seconds spent resting (in grey) and spent grazing (in black) on each cobble (numbered from 1 to 9) by the dominant and the dominated specimens in the unpolluted tank.

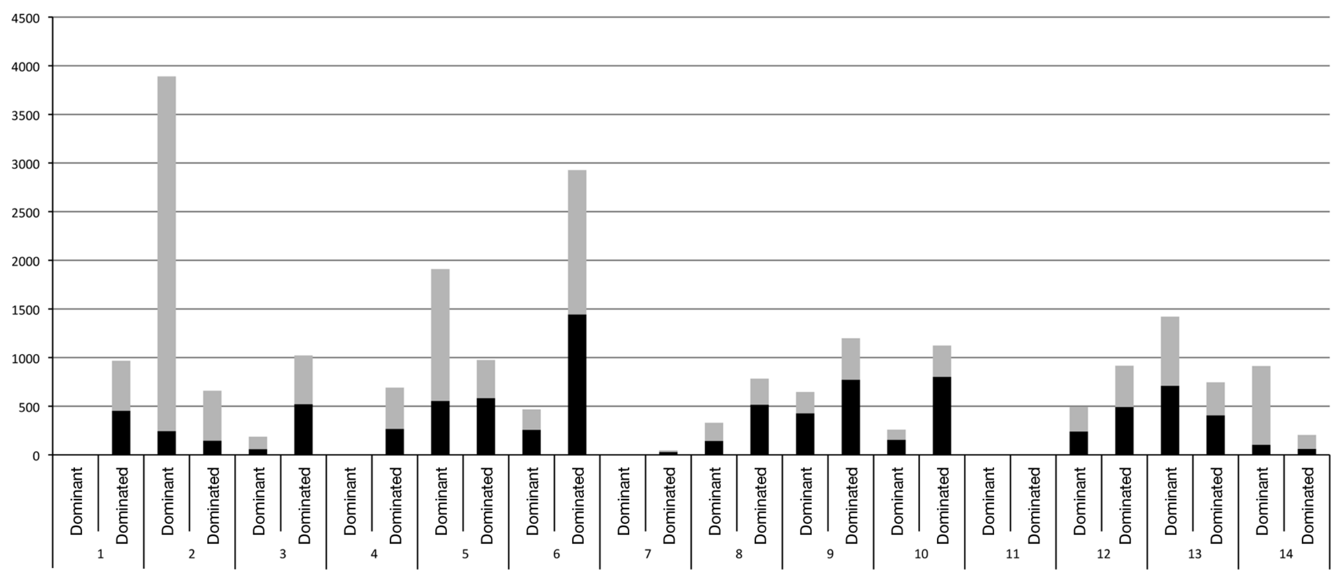


Figure 3. – Time in seconds spent resting (in grey) and spent grazing (in black) on each cobble (numbered from 1 to 14) by the dominant and the dominated specimens in the polluted tank.

Table I. – Wilcoxon test comparing the time spent grazing between dominant and dominated specimens and between tanks (italic: unpolluted tank; bold: polluted tank.).

Wilcoxon test	**Dominant grazing time**	**Dominated grazing time**	**Total grazing time**
Dominant grazing time	0.73		
Dominated grazing time		0.12	
Total grazing time			0.52

Comparison of the time spent grazing between dominant and dominated

A Wilcoxon test showed that in both tanks, there was no difference in the time spent grazing between dominant and dominated specimens (W = 0.45 for the non-polluted tank and W = 0.42 for the polluted tank).

Comparison of the time spent grazing between dominant and dominated specimens and between tanks

A Wilcoxon test showed that there is no difference in the time spent grazing between the polluted and the non-polluted tanks (Tab. I).

BenthoTorch data

We used a BenthoTorch to measure biofilm composition in green algae, cyanobacteria and diatoms at the end of the experiment (Tab. II).

Green algae that develop in polluted conditions are filamentous. Julius *et al.* (2005) proposed a representation of the benthic algal community succession on the river substrate. First colonizers are characterised by colonies of tuft forming diatoms species and motile stalk growing forms. The development of stalk forming species continues until is reaches a dominant phase of stalk growing species. Finally, the macrophytes develop, supporting numerous epiphytic species of diatoms. This could explain the higher concentration of diatoms and cyanobacteria in the polluted tank, the macrophytes offering a substantial additional surface area. Watson (2000) wrote that in the *Sicydium* genus there were stone biting species, such as *S. plumieri*, and algae eating species such as *S. punctatum*. According to him, stone biting species have a feeding habit of scraping algae from rock surfaces, while the algae eating species feed on filamentous algae and other soft vegetation. However, more recent studies (Monti *et al.*, 2010; Keith *et al.*, 2015), show that herbivorous Sicydiinae gobies like *Sicydium* and its sister genus *Sicyopterus* Gill 1860 are incapable of eating filamentous algae. *Sicydium* species are anatomically incapable of scraping diatoms off the macrophytes, they are adapted to grazing biofilm off hard surfaces such as rocks. Indeed, *Sicydium punctatum* has upper jaw teeth entirely tricuspid with the outer cusps rounded and the central cusp pointed and smaller than those laterally with heavy dental reinforcement at base of all 3 cusps (Watson, 2000). This dentition is comparable to that of the sister genus, *Sicyopterus*, which feeds by scraping the biofilm off hard surfaces (Keith and Lord, 2011; Keith *et al.*, 2015). Also, while observing the fish, both in the tank and *in vivo*, they were always seen scraping the rocks, and never the macrophytes.

Correlation between time spent grazing and biofilm composition

Spearman correlation tests were undertaken on both tanks to see whether there was any correlation between the biofilm composition in each tank and the time spent grazing by the fish (Tab. III).

Spearman correlations show that there is a positive correlation in both tanks between the amount of cyanobacteria and the total time spent grazing, showing that cyanobacteria might be part of *Sicydium* diet. In the unpolluted tank there is a positive correlation between the amount of diatoms and the time spent grazing, but this correlation disappears in the polluted tank; the presence of green algae might be preventing *Sicydium* from accessing the diatoms, although present in higher concentration. Also, when there is a positive correlation and in both tanks, the relation is more significant for the dominant specimen, showing that the dominant males maintain the gardens.

Diatom composition of the biofilm and the digestive tracts

There seems to be no difference in the diatom composition between grazed and ungrazed zones of the unpolluted tank (Fig. 4). The dominant diatom species found in the biofilm is *Eolimna sp6*. This species is found in the DT, although it isn't the most frequently found species. Indeed, the most frequent ones found in the DT are *Achnanthidium subhudsonis* and *Eolimna ruttneri*. These two species are more numerous in the grazed zone compared to the ungrazed zone, suggesting an enhanced growth of these preferred diatoms by the grazing activity. The lack of replicates doesn't allow us to statistically analyse the results, but these first descriptive results lead to think that the fish might prefer to feed on specific diatom species and that they sustain the growth of the species by their grazing activity. We can then support the hypothesis of a "gardening behaviour", as explained by (Fitzimons *et al.,* 2003).

For the polluted tank, only the biofilm sample was analysed, as the DT sample was unfortunately not exploitable as the concentration of diatoms in the pooled DT was too low to be analysed, and the frustules that were present were too deteriorated to allow identification. We thus couldn't compare the diets between the two conditions. The diatom

Table II. – Mean biofilm composition in each tank measured with the BenthoTorch. Results are given in μg chla.cm^{-2} ± standard deviation.

	Green algae	Diatom	Cyanobacteria
Polluted tank	0.98 ± 0.84	2.27 ± 1.59	0.65 ± 0.38
Unpolluted tank	0	0.46 ± 0.28	0.41 ± 0.27

Table III. – Spearman correlations. Polluted and unpolluted tanks. *: indicate statistically significant results; NS: non significant; NA: not applicable.

	Green algae (μg Chla.cm^{-2})		Cyanobacteria (μg Chla.cm^{-2})		Diatom (μg Chla.cm^{-2})	
Polluted tank	rho	p-value	rho	p-value	rho	p-value
Dominant grazing time	0.24	NS	0.74	**	0.5	NS
Dominated grazing time	0.13	NS	0.34	NS	0.38	NS
Total grazing time	0.08	NS	0.65	*	0.49	NS
Unpolluted tank						
Dominant grazing time	NA	NA	0.88	**	0.78	*
Dominated grazing time	NA	NA	0.67	*	0.58	NS
Total grazing time	NA	NA	0.95	***	0.87	**

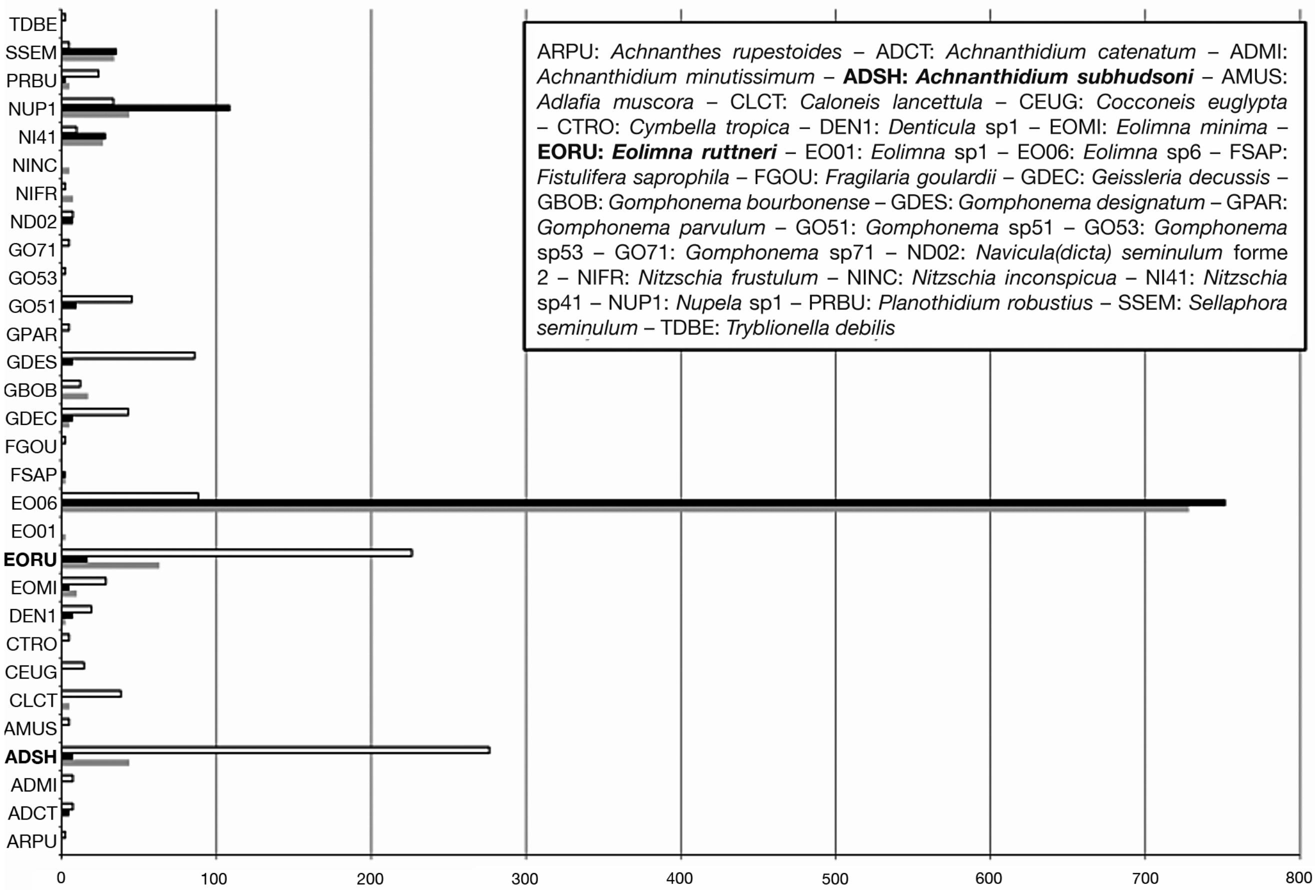


Figure 4. – Diatom composition (expressed as the number of cells counted in the sample) of the grazed (grey) and ungrazed (black) cobbles and the digestive tracts of *Sicydium punctatum* (white), in the unpolluted tank.

composition of the biofilm, like in the unpolluted tank, was the same between the grazed and ungrazed zones (figure not shown). We therefore compared the overall diatom composition between the polluted and unpolluted tanks. We added the number of diatom species found in the grazed and ungrazed samples for each tank and compared the results between the two tanks (Fig. 5).

The species richness is slightly higher in the polluted tank than in the unpolluted tank (24 *vs*. 20 species), which is not a clear difference, so one can say that the species richness is comparable in both tanks. One of the preferred diatom species (*A. subhudsonis*) is in greater number in the unpolluted tank, while the other, *E. ruttneri*, develops well in the polluted tank. The attached diatom communities may be classified according to their substrata and there are differences between epilithic and epiphytic assemblages but there are overlaps between these communities in the species they contain (Round *et al.,* 1990). *E. ruttneri* and *A. subhudsonis* are distinct life-forms species: the first one is motile and the second one is a low profile diatom but as both epilithic and epiphytic communities contain similar life-forms, this is not helpful. Some species (*i.e. Cocconeis pediculus, Cocconeis placentula, Lemnicola hungarica*) are mostly found as an epiphyte (Taylor *et al*., 2007). Some species are even known to be specific of some plants species (Round *et al*., 1990) but this is not the case of *E. ruttneri* and *A. subhudsonis* that can be considered as mostly epilithic. The green algae probably offers more surface area for the growth of diatoms, but by preventing grazing on the rocks, it might be preventing "cultivation" of *S. punctatum*'s preferred species. The green algae developing in polluted conditions may thus have a negative effect on the food resource of *Sicydium*, although it is still present, it is inaccessible. Furthermore, *A. subhudsonis* is a solitary stalk forming species, and *E. ruttneri*, is a solitary moving cell, both of them probably developing at the beginning of the benthic algal community succession. Both the gardening behaviour and the growth of macrophytes can influence the diatom biodiversity through different mechanisms. But, by regularly grazing the rocks, the fish continuously keep the substrate in a pristine state, favouring primary succession species, and thus its favoured species. Gut contents of post-larvae, juvenile, and adult specimens of

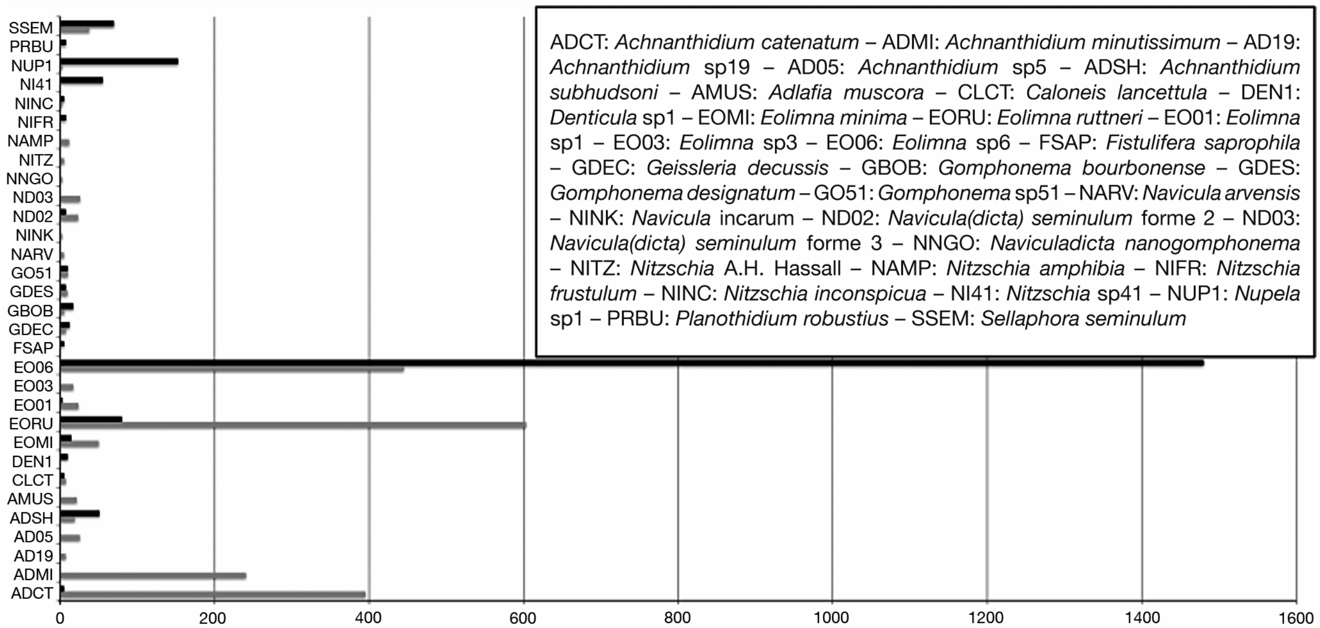


Figure 5. – Diatom composition (expressed as the number of cells counted in the sample) from the biofilm in the unpolluted (black) and the polluted (grey) tanks.

the Hawaiian gobiid fish *Sicyopterus stimpsoni* (Gill, 1860) were examined to catalogue the periphyton ingested by this species (Schoenfuss *et al*., 2004; Julius *et al.,* 2005; Sherwood and Nishimoto, 2005). Benthic diatoms found with juvenile and adult fish assemblages represented multiple genera that live in a narrow set of environmental conditions. These diatoms grow during the specific period of biofilm colonization during the development of the benthic community in Hawaiian streams. This suggests a highly specialized dietary behaviour that depends heavily on continually restarting the benthic periphyton succession pattern, which appears to be regulated by the hydrological cycles of streams on the island (Julius *et al*., 2005).

Fitzsimons *et al*. (2003) have shown that *S. stimpsoni* from Hawaii maintained 'gardens' of low-growing periphyton in swift water on the upper surfaces of large pebbles and boulders. These conspicuous patches of diatoms and short filaments of cyanobacteria represent a food source and the area for the initiation of stereotypical social behaviour, including territoriality and courtship. In this work, we show that the same behaviour most undoubtedly also occurs for *Sicydium punctatum* (this work; Monti *et al*., 2010).

CONCLUSION

In this preliminary study, the first hypothesis that we put forward in the introduction [(i) the pollutant induces changes in the gardening behaviour] was not validated; the social behaviour of the fish doesn't seem altered by the pollution. The second one was however validated [(ii) the "gardens" are mainly maintained by dominant males]. Indeed, in both tanks, fish show the same social behaviour; with a dominant male guarding its territory. As the dominated specimens graze practically exclusively on the dominant's preferred areas, and as there is a strong positive correlation between the time spent grazing by the dominant and the biofilm composition, we can conclude that there is a gardening behaviour and that it is carried out by the dominant male. The dominant male seems to be maintaining a "garden" from which other fish on the territory can benefit. As for the third hypothesis [(iii) diatoms found in the epilithic biofilm are the main source of food influencing grazing], we found that diatoms are indeed a source of food, as they are found in the digestive system, but cyanobacteria also seem to be a source of food influencing grazing. In the polluted tank, the growth of green algae might prevent the fish from grazing the diatoms off the rock surfaces, and the growth of preferred diatom species in the diet of *Sicydium punctatum* is altered. The pollutant may alter biofilm composition and fish may have to shift diet to other more present elements of the biofilm. Also, the presence of the pollutant may favour the growth of green algae, which are not part of the *Sicydium* diet, as green algae are filamentous and *Sicydium* teeth are not adapted to the grazing of filamentous algae. This study gives preliminary results concerning the gardening behaviour. The same analysis should be undertaken *in vivo* by spotting a territory and by creating a protected patch in order to compare grazed and untouched biofilm composition in polluted and pollutant free areas. Taxonomic analysis of the biofilm should also

be undertaken at different time of the experiment to measure biofilm evolution in time and measure the effect of continuous grazing on a specific patch.

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